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Supplementary Information for

Multipotent fetal-derived Cdx2 cells from placenta regenerate the heart

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Supplementary Information Text

Methods:

Mice:

CDX2-cre mice were hemizygous with a 9.5 kb human Caudal type homeo box 2 (*CDX2*) promoter/enhancer sequence directing expression of a nuclear-localized Cre recombinase. The ROSA $^{nT\text{-}nG}$ allele consists of a CMV enhancer/chicken beta-actin core promoter, a *loxP*-flanked nT cassette, and an nG cassette, all inserted into the Gt (ROSA) 26Sor locus. Prior to exposure to Cre recombinase, red fluorescence was noted in the nuclei of cells/tissues. No eGFP expression was reported prior to *cre*-mediated recombination in this mouse model. When bred to Cre recombinase expressing mice, the floxed nT cassette is deleted in the *cre* expressing cells/tissues of the offspring allowing the expression of the downstream nG cassette resulting in strong nuclear green fluorescence of *cre* expressing cells and future cell lineages derived from these cells. This strategy enabled the labeling of fetal Cdx2 cells in placenta with eGFP and in the F1 progeny, Cdx2 lineage cells will be labeled with eGFP. This transgenic approach leverages the expression of recombinase to activate the expression of reporter genes and once cre is activates within the cell, all progeny will express the eGFP fluorescence. Mice were housed under conventional conditions, fed standard chow and water *ad libitum.* All animal care complied with the *Guide for the Care and Use of Laboratory Animals* by the US National Institutes of Health, and institutional guidelines at Mount Sinai School of Medicine.

Randomization, Blinding and Replication

In myocardial infarction experiments, after MI, wild type male mice were randomized to receive either PBS vehicle injection or Cdx2-eGFP cell injection (**Fig. 6***A*) or eGFP**-** cell injection in some experiments. Animals were blindly randomized after MI to receive PBS control or Cdx2-eGFP cells. Investigators who were blinded to the intervention in the study performed data collection and analysis. MRI analysis was performed by a cardiac MRI specialist who was only given number code for mice and completely blinded to treatment versus control animals. All the experiments were replicated more than three times in identical conditions. Biological and technical replicates are indicated in the figure legends wherever appropriate.

Guidelines:

All animal care complied with the *Guide for the Care and Use of Laboratory Animals* by the US National Institutes of Health, and institutional guidelines at Mount Sinai School of Medicine.

Processing of end-gestation placenta and Cdx2-eGFP cell isolation

Pregnant female mice were euthanized (both wild type and transgenic) on embryonic day 18 (e18) and placentas were isolated and processed to yield single cell suspensions The isolated placentas were rinsed in 1X HBSS to remove traces of maternal blood and tissues. Placentas were then minced and digested using a combination of collagenase-II 1.5mg/mL (Worthington Biochemical Corporation, Lakewood, NJ, USA) and pronase 1mg/mL (Calbiochem, Massachusetts, USA) enzymes at 37 °C for 30 minutes. Red blood cells were lyzed using ammonium chloride based lysis solution (8.26 g NH4Cl, 1g KHC03, 0.037g EDTA) for 15 min in dark at room temperature. The cells were washed and suspended in 1X HBSS and were sorted to collect eGFP cells through the BD influx cell sorter (BD Biosciences, San Jose, CA). e18 placenta cells from wild-type mice served as negative control for eGFP fluorescence. eGFP negative cells are tdTomato+ based on the transgenic strategy and were further purified based on the lack of CD9 expression. Cdx2 interact with CD9 (1) and we have observed that isolated murine placental Cdx2-eGFP cells were homogenously CD9 positive. Therefore the sorted tdTomato+ cells (Cdx2-eGFP-) were further depleted for CD9 (tdTomato+CD9- cells).

Processing of whole mouse embryos and embryonic tissues

Embryos were separated from the placenta and was perfused using PBS before paraffin embedding and processing to yield sagittal sections. Additionally, embryonic tissues were isolated from the embryos by visualizing under dissection microscope. The tissue sections and the embryo sections were deparaffinized and subjected to rehydration and auto fluorescence was quenched using 0.7% sudan black B in 70% ethanol. The sections were subsequently stained using DAPI and was observed under Ziess epifluorescence microscope with Axiovision software. Whole embryo sections were scanned using Panaromic Scanner 20X with digital immunofluorescence to visualize DAPI and endogenous eGFP fluorescence.

Cardiomyogenic differentiation of Cdx2-eGFP cells *in vitro*

Glass bottom 24 well plates were coated with 1µg/ml laminin (Corning, Bedford, MA, USA) and neonatal cardiomyocyte feeder layers were prepared as previously described (2). Sorted Cdx2-eGFP cells were seeded and cultured using Iscoves DMEM (Corning Cellgro, Manassas, VA) containing 10% FBS, L-Glutamine, 25mM HEPES and 1x Penicillin and Streptomysin. Cells were monitored for a period of over four weeks and live cell imaging was done to capture spontaneous beating using Zeiss Axiovision Observer Z1 inverted fluorescent microscope equipped with Axiovision software (Carl Zeiss, Munich, Germany). In parallel experiments, sorted Cdx2-eGFP cells were seeded on mitotically inactivated adult cardiac fibroblasts (Cell Biologics, Chicago, IL, USA). Briefly the cardiac fibroblasts were grown to confluence in complete fibroblast medium (Cell Biologics, Chicago, IL, USA) and were then treated with 10µg/mL of Mitomycin C (MP Biomedicals, Solon, OH, USA) for two hours at 37°C to inhibit fibroblast cell growth. The feeder cells were then washed thoroughly with 1X HBSS to eliminate excess mitomycin C and the medium was replaced with fresh 10% Iscoves DMEM.

XY chromosome analysis

Mouse XY chromosome probes were purchased from Empire Genomics (Empire Genomics LLC, Buffalo, NY, USA). Slide preparation and hybridization was carried out as per manufacturer's instructions and cells (separated by trypsinization from the co-culture) were mounted on coverslips using Dapi/Antifade. Images were captured using Zeiss Axioplan 2 fluorescence microscope equipped with CytoVision software (Genetix Corp, San Jose, USA). The filter range used was X probe – green-dUTP- 520nm emission maximum, Y probe- red-dUTP- 603nM emission maximum

Clonal expansion and differentiation of Cdx2-eGFP cells

Single Cdx2-eGFP cells were sorted onto glass bottom 96 well plates containing Mitomycin C treated adult CF-feeders with complete medium. Cells were monitored daily for a period of 10 days. Images of clonal proliferation were captured using Zeiss Axiovision Observer Z1 inverted fluorescent microscope (Carl Zeiss, Munich, Germany) using Axiovision software. For analysis of clonal differentiation, single eGFP+ and eGFP**-** cells were plated on both CF and CM feeders and were maintained until 5 weeks in culture before the cells were fixed, permeabilized and immunostained for cardiac

(cTnT) and vascular (CD31-endothelial and α-SMA- smooth muscle cells) lineage commitment.

Transwell migration assay

Chemotactic responses of isolated Cdx2-eGFP cells were studied using a transwell migration experiment. 24 well compatible transparent PET membrane 8 micron inserts (Thermo Fisher Scientific, Nazreth, USA) were used and equal number of Cdx2-eGFP cells was added to the upper wells. The lower wells contained Iscoves DMEM +2% FBS as medium with and without 100ng of SDF1α. For blocking experiments, 1μM AMD3100 was used to pretreat Cdx2-eGFP cells and the migration to SDFα was compared to the untreated eGFP cells. The cells were incubated at 37°C and 5%CO_2 for 3 hours. Following incubation, the inserts were carefully removed and the migrating eGFP cells were imaged in each set. The migrating and non-migrating cells were counted, and percent migration was calculated as migrating cells/total cells x 100.

Proteomic analysis

Cdx2-eGFP cells were isolated from the e18 placentas of three different mice and the cells were pelleted and snap-frozen. Pellets were lysed in 100μL of freshly made 8M urea, dissolved in 100mM Tris-HCl (pH 8.5), containing phosphatase and protease inhibitor. Lysates were ultrasonicated to complete lysis and to shear DNA (5 successive 10sec pulses). After determining protein concentrations using a Bicinchonic Acid (BCA) assay, samples were reduced and alkylated in (tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and fresh Iodoacetamide (IAA), and diluted to 500uL. Samples were digested at 37°C overnight with constant agitation using Trypsin/LysC mix at a 40:1 ratio. Digests were acidified with Trifluroacetic acid (TFA), desalted on a 96-well HLB micro elution plate, and dried to completion prior to mass spectrometry (MS) analysis. Samples were suspended in 0.1% FA at 1μg/μL. 6 μL of samples were injected onto a C18 μPLC column and analyzed under Data Dependent Acquisition mode using an Orbitrap Elite mass spectrometer (Thermo Fisher, USA). Average fold-change values were inferred from the quantified protein-level values as the quotient of the experimental and control groups. These expression differences were subject to ingenuity pathway analysis (IPA). The purpose of this IPA analysis was to gain preliminary insights into cell

function and processes that maybe reflected by protein expression patterns from the Cdx2-eGFP cells relative to the control ES cells that do not express Cdx2.

Immunofluorescence and flow cytometry

Wild-type placenta cells were cytospun (Shandon, USA) onto slides and fixed using 4% paraformaldehyde (PFA) for 15 min at room temperature (RT). The slides with smears were then permeabilized using 0.25% Triton X 100 in 1X HBSS for 20 min at RT, followed by blocking using 5% Bovine Serum Albumin (BSA) in 1X HBSS for two hours. Cdx2 monoclonal antibody (CDX-88, Biogenex) was added at a 1:100 dilution overnight at 4 °C, followed by donkey anti-mouse Texas red secondary antibody (Thermo scientific, IL, USA) at 1:200 dilution for 1 hour at RT. The slides were then rinsed three times using 1X HBSS and counterstained with DAPI and cover slipped with mounting media (KPL, Gaithersburg, MD, USA) and the fluorescence was visualized using Zeiss AxioVision Observer Z1 inverted fluorescent microscope (Carl Zeiss, Munich, Germany) using Axiovision software. Similarly after co-culture experiments, the cells were fixed, permeabilized and blocked as described above. In the MI model, hearts were excised 3 months post-treatment and fixed in 4% PFA. Paraffin embedded sections were blocked and incubated with antibodies. For flow cytometry analysis, MI hearts from control and Cdx2 group were excised and enzymatically digested using a mixture of collagenase and pronase followed by RBC depletion and subsequent flow cytometry analysis. Other tissues were manually processed, RBC depleted and analyzed by flow cytometry. Gating strategy for the population was set on control mice cells and applied appropriately to the test group in all tissues. For immunofluorescence analysis *in vitro* and *in vivo*, primary antibodies including anti- GFP Alexa flour 488 antibody (Molecular probes, Oregon, USA), goat polyclonal cardiac troponin T (Santa Cruz Biotechnology Inc, USA), mouse monoclonal α -sarcomeric actinin (Thermo Scientific, USA), rabbit polyclonal sarcomeric actinin (Santacruz Biotechnology Inc.), rat polyclonal anti-connexin 43, rat anti-mouse CD31 (BD biosciences San Jose, USA), goat anti-rat/mouse Tie-2 (Biotechne Inc), mouse monoclonal αSMA (Sigma Aldrich, USA), mouse monoclonal smoothelin (Invitrogen), goat polyclonal Myh11 (Themo Scientific, USA) were added and incubated overnight at 4°C. Donkey anti -goat Texas red IgG (Thermo Scientific, USA) and donkey anti-mouse Alexa flour 647 (eBioscience, San Diego, CA) ,

Donkey anti-rabbit Cy5 (MilliporeSigma, St. Louis, MO, USA), goat anti-rabbit alexafluor 488 (Invitrogen, USA) secondary antibodies were used and further incubated for 1hour at RT. Cells were washed three times. To reduce autoflourescence, the cells were then treated with 0.7% Sudan Black B (Acros Organics, NJ, USA) in 70% ethanol for 10 minutes. The cells were washed extensively using 1X HBSS and counter stained with DAPI and visualized using Zeiss Axiophot2 Fluorescence Microscope (Carl Zeiss, Munich, Germany) using Axiovision software. *In vitro* and *in vivo* quantification of cellular differentiation was carried out by selecting random region of interest in each well (ROI) and for post MI heart sections, the infarct (injury) zone, border zone and distal zone were demarcated and ROIs were selected using Axiovision software. Z stack analysis (63X magnification) were performed on selected fields to confirm that eGFP/DAPI co-localizes and is embedded within the cells positive for sarcomeric actinin in post-MI heart. For the clonogenic culture images, scale bars have been reconstructed based on the original scales for adequate visibility. For MHC class I and class II (eBioscience, San Diego, CA, USA) proteins, sorted Cdx2-eGFP cells were fixed in 1% PFA and blocked using mouse Fc blocker (CD16/32- BD Biosciences) in FACS buffer (0.1% BSA in 1x HBSS) followed by antibody incubation at 4°C for 40 min. Data analysis was performed using BD FACS Diva software (BD, Biosciences).

Myocardial Infarction and Cdx2 cell injection

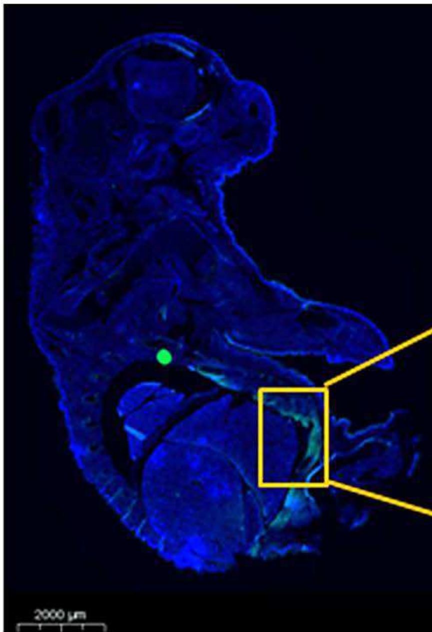
Briefly, the mice were anesthetized using intraperitoneal injection of Ketamine (100mg/kg)/Xylazine (5mg/Kg), followed by endotracheal intubation. Chest cavity was opened and after careful dissection of pericardium, LAD was ligated using 9-0 nylon suture. The wound was closed using 6-0 sutures and post removal from intubation mice were allowed to slowly recover in a warm atmosphere (delta phase Isothermal pads-Braintree Scientific Inc, MA) and were monitored closely. Pre-operatively and post–operatively (for 3 days), 0.05mg/kg of buprenorphine was intraperitoneally administered as an analgesic regimen. Cardiac MRI was carried out 1 week post-MI and Cdx2-eGFP cells were intravenously delivered through lateral tail vein.

RNA extraction, cDNA preparation and PCR

Total RNA was extracted from WT placental cells and sorted Cdx2-eGFP cells using Tri reagent (Thermo Fisher Scientific, USA) followed by Pico pure RNA isolation Kit

(Thermo Fisher Scientific) according to manufacturer's instructions. Eluted RNA was subjected to DNase I digestion (Ambion, Thermo Scientific, USA) to eliminate residual genomic DNA. 1µg of RNA from WT mice placental cells was reverse transcribed to cDNA using Maxima first stand cDNA synthesis kit (Thermo Fisher Scientific, USA) adhering to manufacturer's instructions and standard PCR was run using specific primers for murine Cdx2. For sorted Cdx2-eGFP cells, RNA was pre-amplified and cDNA was prepared using RT2 preamp cDNA synthesis kit (Qiagen, Valencia, CA). Immune profiling for 84 genes was done using mouse innate and adaptive immune response gene array (Qiagen, Valencia, CA) in Step One Plus real time PCR System (Thermo Fisher Scientific, USA). The PCR cycling condition was as follows: 50°C for 2 min (1cy), 95 °C for 10 min (1 Cy), followed by 40 cycles of 95 °C (15 sec) and 60°C (1 min). The results were normalized to the endogenous control genes in the array and were analyzed based on comparative Ct method as described (2).

e18 embryo sagittal section



hind gut

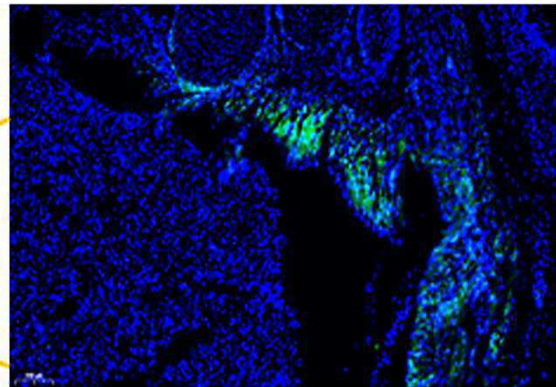


Fig. S1. Cdx2-eGFP expression in e18 embryo

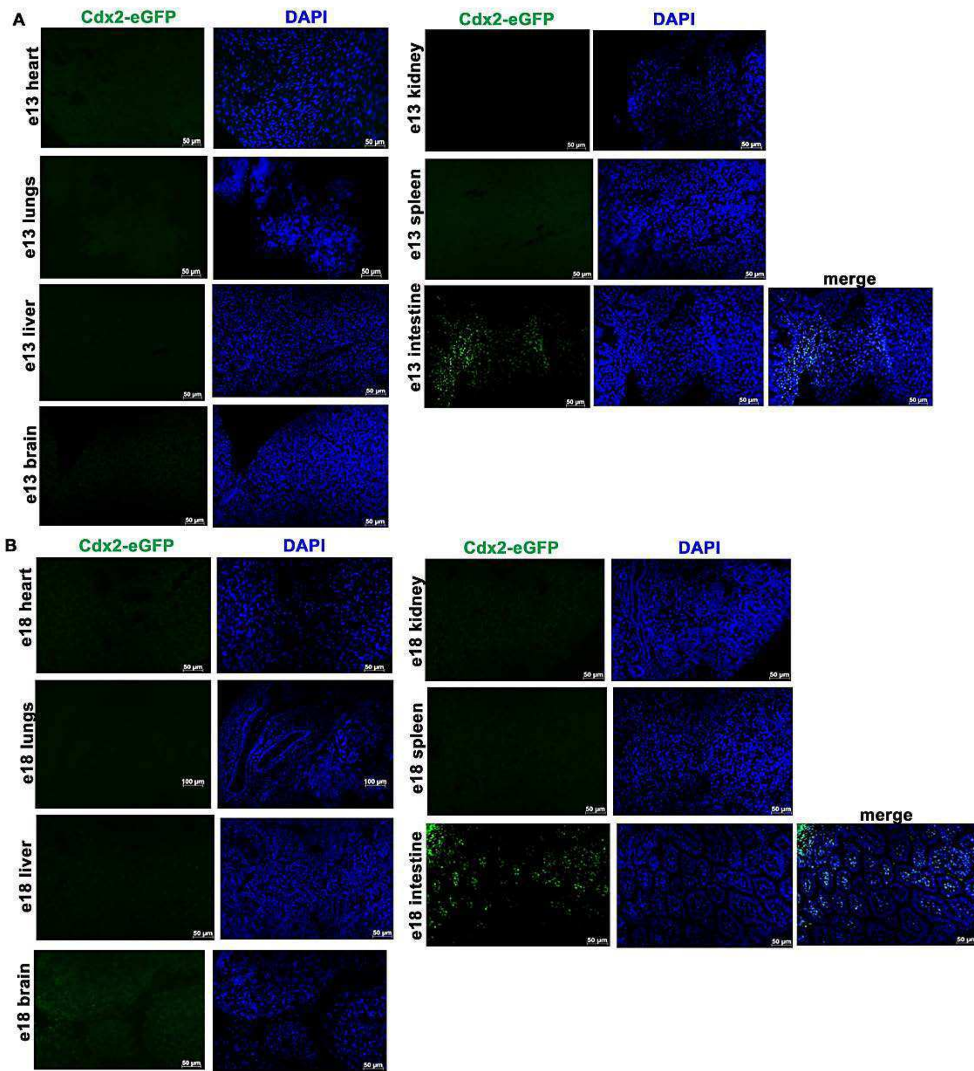


Fig. S2. Cdx2-eGFP expression in e13 and e18 fetal organs

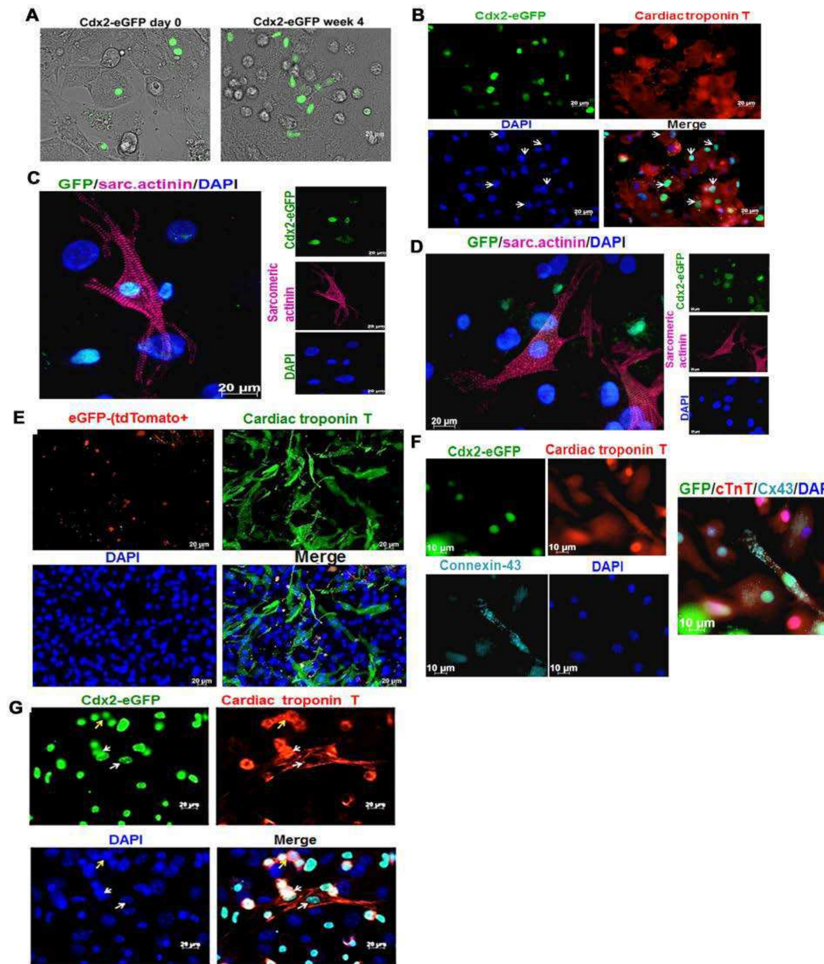


Fig. S3. Cardiac differentiation of Cdx2-eGFP and eGFP- cells *in vitro*

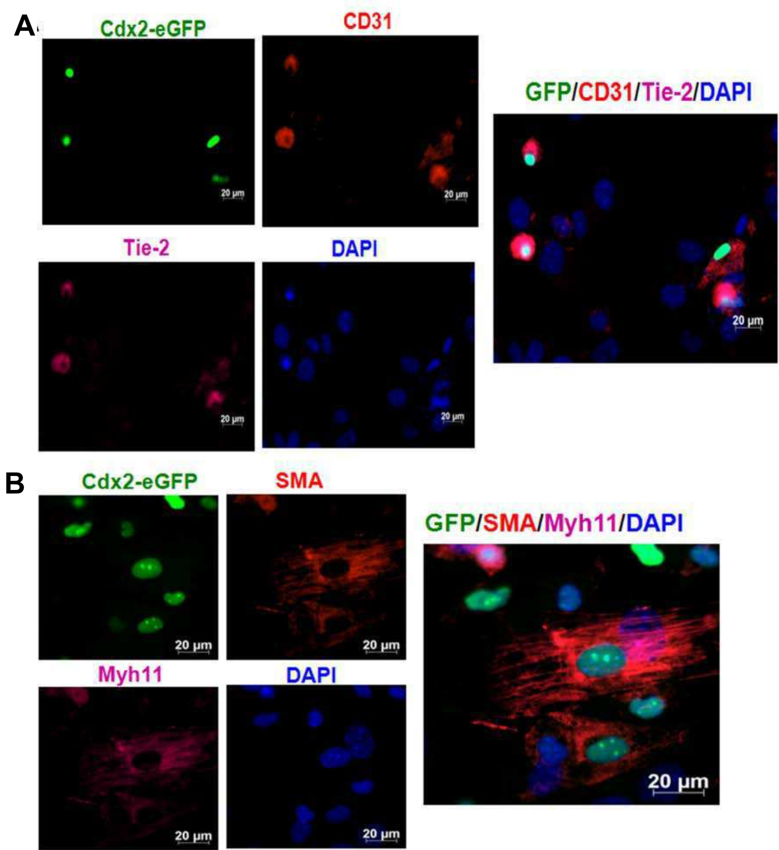


Fig. S4 Vascular differentiation of Cdx2-eGFP cells *in vitro*

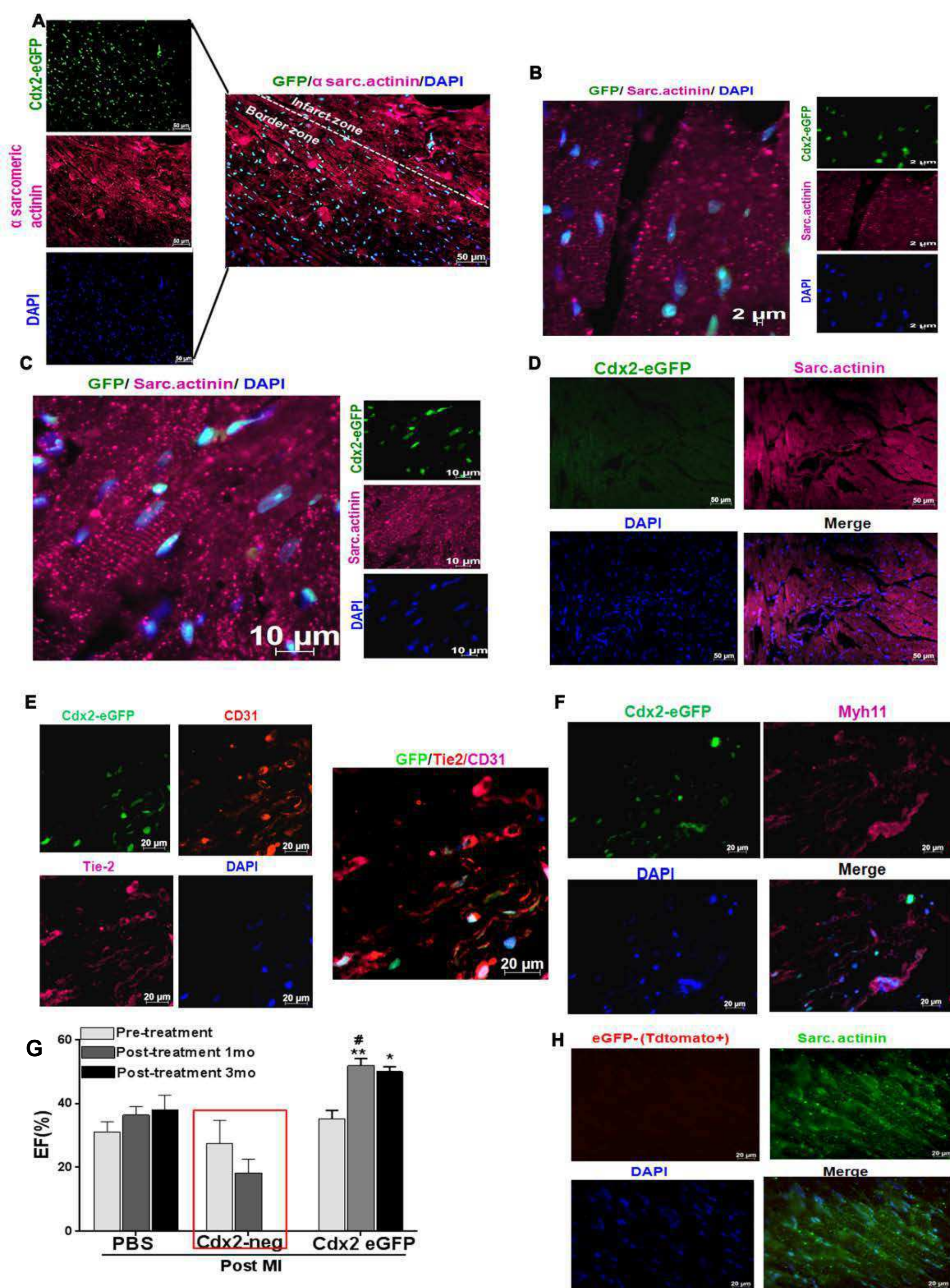


Fig. S5. Cdx2-eGFP cells contribute to cardiac repair *in vivo*

Table S1. Percentage of Cdx2-eGFP cells in e18 placentas

Sample (mouse)	**% Cdx2-eGFP cells from placenta (FACS)**
1	3.8
2	3.7
3	3.56
4	2.68
5	0.82
6	1.74
7	5.89
8	3.68
9	2.10
10	3.91
11	5.11
12	2.5
13	3.27
14	2.39
15	1.73
16	4
Mean ± SEM	**3.18 ± 0.3265**

Table S2. Left Ventricular Ejection Fraction (LVEF) of control (PBS injected) and test (Cdx2-eGFP cell injected) mice before and after treatment post infarction

Control mice post MI	**Pre-treatment**	**1 month post treatment (PBS)**	**3 month post treatment (PBS)**
1	27.67	31	28.6
2	42	46.2	55
3	23	34.4	38.7
4	30.7	34	33
5	33.3	37	36.2
Test mice post MI	**Pre-treatment**	**1 month post treatment (PBS)**	**3 month post treatment (PBS)**
1	30.7	45.97	46.28
2	37.1	50.73	56
3	26.7	43.33	44.04
4	45	54.11	50
5	44.5	57.4	55.8
6	40	47	50
7	37.4	63	51.2
8	26.5	53.6	45.8

Fig.S1. Cdx2-eGFP expression in e18 embryo: A representative whole embryo section from e18 gestation stained using DAPI to visualize the nucleus and any endogenous eGFP fluorescence (no antibody mediated staining was performed for eGFP). The highlighted field depicts the endogenous eGFP fluorescence along the caudal/hind-gut region of the embryo

Fig.S2. Cdx2-eGFP expression in e13 and e18 fetal organs: e13 and e18 embryonic tissues were studied for the presence of endogenous Cdx2-eGFP cells during gestation. (A) and e18 (B) embryonic tissues showing limited or no expression of eGFP fluorescence in all tissues except intestine as shown.

Fig.S3. Cardiac differentiation of Cdx2-eGFP and eGFP- cells *in vitro:* (A) Representative images of Cdx2-eGFP cells on neonatal cardiomyocyte feeders on day 0 and after 4 weeks in culture, showing stable eGFP signal. Images do not represent the same field. Scale bar 20µm. (B) Cdx2-eGFP cells at an early differentiation stage expressing cTnT+ cells (red) within 2-3 weeks on neonatal CM feeder (C-D) Cdx2-eGFP cells differentiae into cardiomyocyte of mature appearance within 5 weeks in culture expressing sarcomeric actinin (pink), related to Fig.2F (E) eGFP- (tdTomato- nuclear red) cells did not appreciably differentiate into cardiomyocytes on CM feeders (cTnt+ cells-green), DAPI (nuclei-blue), scale bar 20µm. (F) Early differentiating Cdx2-eGFP cells expressing connexin-43 within the cell, related to Fig.2I (G) Cardiac differentiation of Cdx2-eGFP cells in CF feeders. White and yellow arrowheads indicate late and early differentiation, respectively, showing different cell morphology of the cells with nuclear eGFP. Scale bar 20µm.

Fig.S4. Vascular differentiation of Cdx2-eGFP cells in vitro: (A) Cdx2-eGFP cells co-express endothelial marker CD31 and Tie-2 in culture with CF feeders in vitro, related to Fig. 3A. (B) Smooth muscle cells expressing SMA and smooth muscle specific Myh11 derived from Cdx2-eGFP cells (CD31/SMA-red, Tie-2/Myh11- pink, DAPi-blue nuclei), Scale bar 20µm.

Fig. S5 Engraftment and differentiation of Cdx2-eGFP cells in border zone of injured myocardium: (A) Engraftment of Cdx2-eGFP cells in the peri-infarct (border zone) region, scale bar 50µm, 20X magnification. Heart sections were immunostained with anti-α sarcomeric actinin (Alexa 647-panel A nuclei DAPI (blue). (B-C) Cdx2-eGFP cells differentiating into cardiomyocyte showing sarcomeres (sarc. actinin-pink) in the border zone of post MI myocardium, sale bar 10µm, 40x magnification, related to Fig 6*F-H*) (D) Lack of homing of Cdx2-eGFP cells into the non-injured (sham) heart *in vivo,* scale bar 50µm, 20X magnification. (E) CDx2-eGFP derived CD31/Tie-2 expressing endothelial cells in the border zone of injured myocardium, (CD31-red), DAPI (blue), scale bar 20µm, related to Fig. 6*I.* (F) CDx2-eGFP derived smooth muscle cells expressing Myh11 in the border zone of injured myocardium, (Myh11-pink), DAPI (blue), scale bar 20µm (G) Intravenous delivery of eGFP**-** cells (as highlighted in the center) did not augment cardiac function as assessed from the ejection fraction after cardiac MRI I mo post-MI, n=4) (H) Heart sections of mice receiving eGFP**-** (Tdtomato+) cells did not show the presence of any Tdtomato-derived cardiomyocytes as shown, sarc. actinin (alexa 488-green, nuclei-DAPI, Tdtomato-red), scale bar 20µm .

Legends for Movie Files - Please Note: Videos should be viewed on 'loop' mode

Movie S1. Live imaging of a spontaneously beating clonally derived Cdx2-eGFP (nuclear eGFP) cells in syncytium with the neighboring feeder layer (non-eGFP)

Movie S2. Live imaging demonstrating lack of spontaneous beating of clonal eGFP**-** (tdTomato+) cells on CM feeders.

Movie S3-S4. Live imaging of a spontaneously beating Cdx2-eGFP (nuclear eGFP) derived cardiomyocyte in syncytium with the neighboring feeder layer (non-eGFP)

Movie S5-S6. Live imaging demonstrating lack of spontaneous beating in eGFP**-** (tdTomato+) cells on CM feeders.

Movie S7. Cardiac MRI representing mid-ventricular short axis view of a normal healthy mouse heart

Movie S8. Cardiac MRI representing mid-ventricular short axis view of control mouse heart. Post MI pre-treatment

Movie S9. Cardiac MRI representing mid-ventricular short axis view of control mouse heart. Post MI, 3 month post-treatment (PBS injection), *same mouse as in Movie 8*

Movie S10. Cardiac MRI representing mid-ventricular short axis view of test mouse heart. Post MI pre-treatment

Movie S11. Cardiac MRI representing mid-ventricular short axis view of test mouse heart. Post MI, 3 month post treatment (Cdx2 cell injection), *same mouse as in Movie 10*

Movie S12. Z-stack analysis of Cdx2-eGFP derived α-sarcomeric actinin+ cardiomyocyte with eGFP nucleus embedded within the α-sarcomeric actinin+ cell, related to Figure 6*F*

Movie S13. Z-stack analysis of Cdx2-eGFP derived cardiomyocyte with sarcomeres and eGFP nucleus embedded within the α-sarcomeric actinin+ cell, related to Figure 6*G*

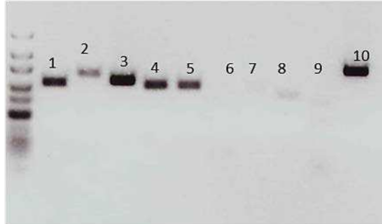
Movie S14. Z-stack analysis of Cdx2-eGFP derived cardiomyocyte with sarcomeres and eGFP nucleus embedded within the α-sarcomeric actinin+ cell- related to Figure. 6*H*

Additional data table S1 (separate excel file)

Excel file related to Fig.5 proteomics

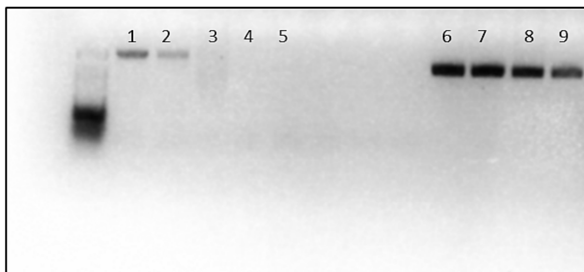
Gel micrographs related to Fig.1*A*

Cdx2 mRNA in fetal organs and adult intestine- Gel 1

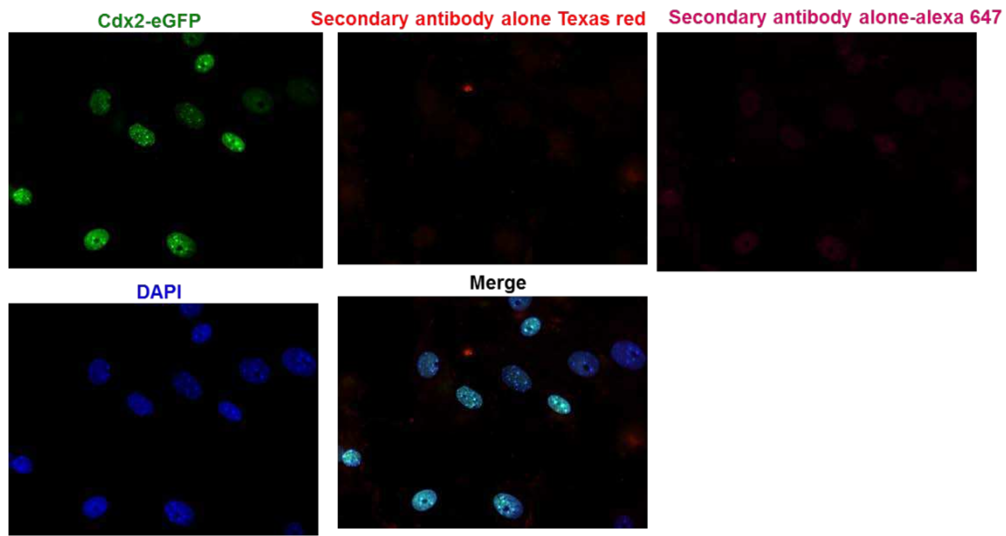


1- **Cdx2 e18 placenta**
2. e18 intestine a
3.e18 intestine b
4. Adult intestine a
5. Adult intestine b
6. e18 heart a
7. e18 heart b
8.e18 heart c
9 - ND
10- gapdh e18 placenta

Gel 2 – Gapdh



1—5 – primers alone –ve control
6- Gapdh e18 intestine
7- gapdh adult intestine
8- **gapdh e18 heart**
9 – gapdh e18 intestine)



Negative control slides for immunofluorescence data in Fig.2*F*

References

1. Boyd M, et al. (2010) Genome-wide analysis of CDX2 binding in intestinal epithelial cells (Caco-2) J Biol Chem 285:25115-25125

2. Kara RJ, et al. (2012) Fetal cells traffic to injured maternal myocardium and undergo cardiac differentiation. Circ Res 110:82-93